

Purification of Interferon γ -Interferon γ Receptor Complexes by Preparative Electrophoresis on Native Gels

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Cytokine-receptor complexes are required for certain studies including crystallization, NMR spectra, and investigation of the biological response mechanism to the cytokine. The purity of the ligand-receptor complex is critical for most of these applications. We investigated the possibility of purifying protein-protein complexes by electrophoresis on native gels. Starting with partially purified mouse and highly purified human proteins, we prepared milligram amounts of interferon γ -interferon γ receptor complexes by preparative electrophoresis on nondenaturing polyacrylamide gels. In both cases, pure ligand-receptor complexes with the correct stoichiometry of binding were recovered. Electrophoresis on preparative native gels may prove to be of general interest for the preparation of protein-protein complexes to be used in diverse studies. © 1993 Academic Press, Inc.

Stable protein-protein complexes, like those between ligands and their receptors, antigens, and antibodies (or F_{ab} fragments), are useful in crystallization studies and in the investigation of the biological response mechanisms to cytokines. Knowledge of the structure of the cytokine binding site would facilitate the design of low molecular weight agonists and antagonists as potential pharmaceutical products against several disorders. The presence of foreign proteins in the desired protein-protein solutions may hinder the formation of diffractive crystals as well as the investigation of biological interactions. The separation of impurities without affecting the desired protein-protein complex is often time-consuming and laborious.

Here we investigated the possibility of producing relatively large amounts of ligand-receptor complexes by using preparative electrophoresis on nondenaturing polyacrylamide gels. Simultaneously, we tried to achieve a significant purification effect, which otherwise would require lengthy chromatographic steps and reduce yields. Electrophoresis on native gels does not affect protein-nucleic acid and protein-protein complexes and is an important analytical tool in the investigation of the interactions between these components (1,2).

The ligands used here were mouse and human IFN γ ³ both produced in *Escherichia coli*. Their species-specific receptors were produced in baculovirus-infected insect cells. The mouse proteins were partially purified (about 50%), whereas the proteins of human origin were highly purified (about 98%). In both cases, we recovered pure ligand-receptor complexes with the expected stoichiometry of binding.

MATERIALS AND METHODS

Materials. Reagents for the preparation of SDS- and native polyacrylamide gels were purchased from Bio-Rad. Low molecular mass size markers were from Pharmacia-LKB.

Analytical methods. The purity of proteins was followed by Coomassie blue R-250 and silver-stained SDS-polyacrylamide gels. The composition of the IFN γ -IFN γ receptor complexes was followed by SDS- and native polyacrylamide gels. The protein concentration and the stoichiometry of the ligand-receptor interaction in the complexes were determined by amino acid analysis (3-5).

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³ Abbreviations used: IFN γ , interferon γ ; IFN $\gamma\Delta 10$, interferon γ lacking 10 residues from the carboxyl-terminal end; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate.

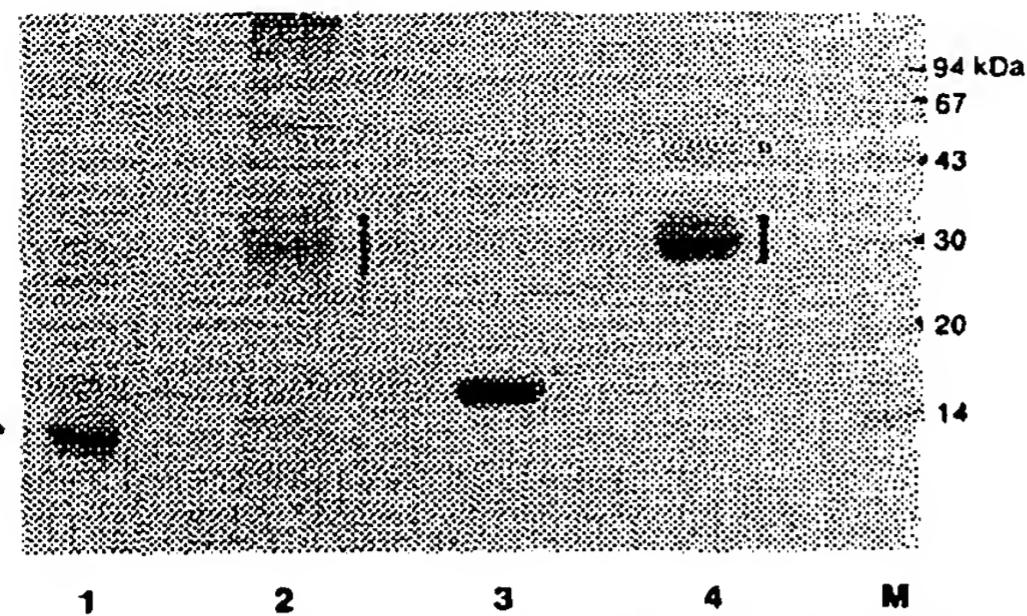


FIG. 1. SDS-PAGE analysis of mouse and human IFN γ s and soluble IFN γ receptors. The proteins (10 μ g each) were electrophoresed on a 13.5% polyacrylamide gel under nonreducing conditions and the gel was stained with Coomassie blue R-250. Lane 1, Partially purified mouse IFN γ . *15-kDa band of IFN γ ; the 14-kDa proteolytic product is not clearly seen. Lane 2, Partially purified soluble mouse IFN γ receptor. The bands corresponding to the glycosylated protein are indicated. Lane 3, Human IFN γ . The band at 32 kDa is the dimeric form of IFN γ . Lane 4, Soluble human IFN γ receptor. The bands corresponding to the heterogeneously glycosylated protein are indicated. *43-kDa impurity. M, Molecular mass markers in kDa.

IFN γ s. Mouse IFN γ was produced in *E. coli* and purified by chromatography on phenyl-Sepharose, hydroxylapatite, and Sephadex G-100 columns. In this study, side fractions derived from the Sephadex G-100 step were used. Human IFN $\gamma\Delta 10$ was also produced in *E. coli* and purified as reported (6).

IFN γ Receptors. Soluble mouse and human IFN γ receptors, composing the extracellular ligand-binding domain of the native proteins, were expressed in insect cells (*Spodoptera frugiperda*). The proteins were secreted into the culture medium and were purified as pre-

viously described (7). The purification involved chromatography on affinity, ion exchanger, and sizing columns. The mouse receptor used in this study was a side fraction of the ion exchange chromatography step. The human receptor was a final purification product. The monoclonal antibodies γ R38 and γ R99 were raised against the native human IFN γ receptor (8).

Polyacrylamide gels. Electrophoresis on SDS-polyacrylamide gels was performed according to Laemmli (9) and on native polyacrylamide minigels according to Wang and Pan (2) with minor modifications. Native separation gels (5%) contained 16.51% acrylamide-bisacrylamide (30:1) solution, 25.01% 960 mM Tris-HCl, pH 8.5, and 58.03% water. Polymerization was initiated by addition of 0.2% Temed and 0.24% ammonium persulfate (10% freshly prepared stock solution in water). The 4% stacking gel contained 13.18% acrylamide-bisacrylamide solution, 24.78% 160 mM Tris- H_3PO_4 , pH 6.9, and 61.45% water. Temed (0.1%) and 0.5% ammonium persulfate (10% stock solution) were added to start polymerization. The anode buffer (lower buffer chamber) contained 65 mM Tris-HCl, pH 7.5, and the cathode buffer (upper buffer chamber) 37 mM Tris-glycine, pH 8.9. The sample buffer contained 10 mM Tris-HCl, pH 7.0, 50% sucrose, and 0.1% bromphenol blue and was mixed with the protein samples at a volume ratio of 5:1. Electrophoresis was performed at 200 V at room temperature.

Preparative electrophoresis. The preparative electrophoresis was performed in a specific apparatus (Bio-Rad, prep cell Model 491) used according to the directions of the manufacturer. Reagents were mixed to prepare 25 ml of 5 or 6% native polyacrylamide separation gel solutions. These solutions were poured into the cylindrical ring of the electrophoresis cell to make cylind-

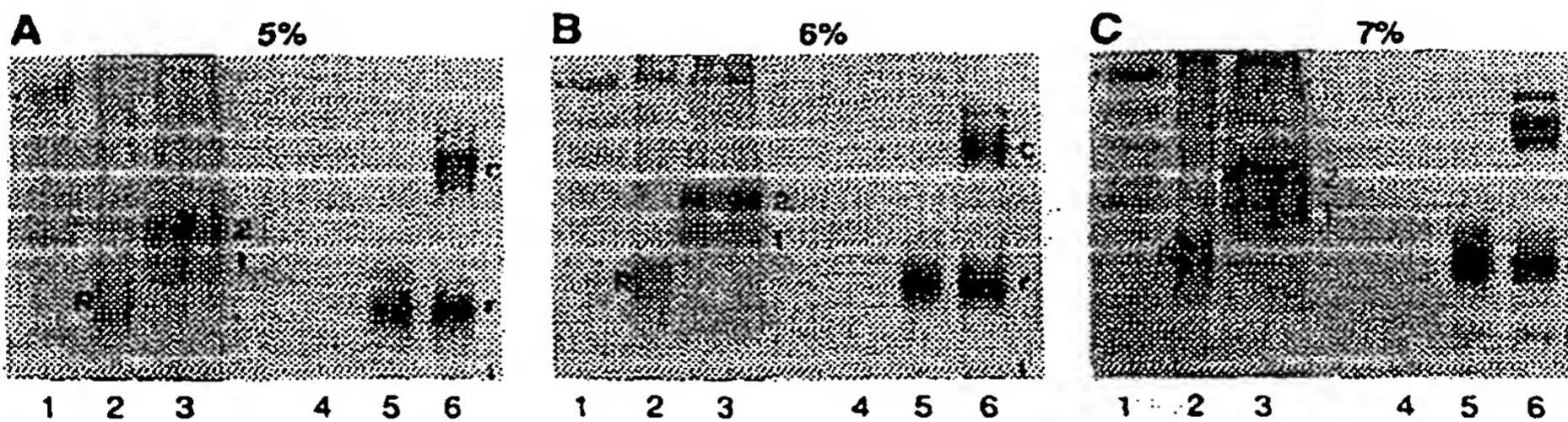


FIG. 2. Analysis of IFN γ -IFN γ receptor complexes on native gels of different polyacrylamide concentrations (A, 5%; B, 6%; C, 7%). In each slot, 5 μ g of protein was applied. The gels were stained with Coomassie blue. (A, B, and C) Lanes 1, Partially purified mouse IFN γ . Band of the full-length protein (■). The other bands represent truncated ligand and impurities. Lanes 2, Partially purified soluble mouse IFN γ receptor. The bands corresponding to the protein are indicated (R). Lanes 3, Mixture of partially purified mouse IFN γ and IFN γ receptor. The bands corresponding to the ligand-receptor complexes are indicated (1, fast migrating complex; 2, slowly migrating complex). Lanes 4, Human IFN γ . The protein was not resolved to clear bands on the native gels. Lanes 5, Soluble human IFN γ receptor (r). The weak band near the front represents the 43-kDa impurity (i). Lanes 6, Mixture of human IFN γ and IFN γ receptor. Band corresponding to the ligand-receptor complex (r). The small band migrating slower than complex c is probably also a ligand-receptor complex. The difference in migration is most likely due to the heterogeneous glycosylation of the receptor. The lower bands represent noncomplexed receptor (r) and the 43-kDa impurity (i).

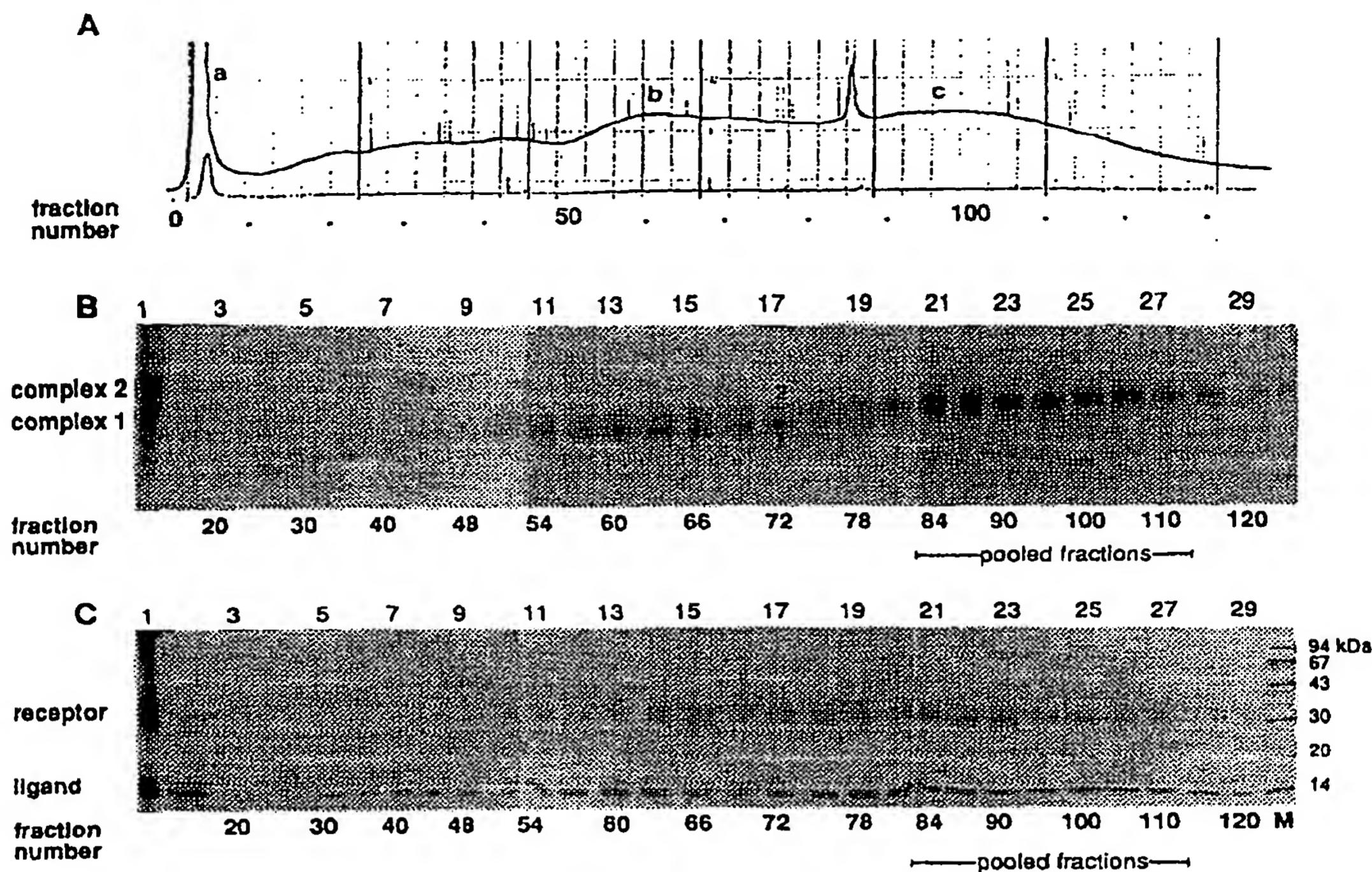


FIG. 3. Absorption profile of the mouse proteins eluted from the preparative nondenaturing gel (A), native (B), and SDS-polyacrylamide gel (C) analysis of the eluted fractions. (A) The absorbance was recorded at 280 nm. The sharp peak between fractions 80 and 90 is an artifact due to a problem with the fraction collector. The protein elution from the preparative gel was interrupted for about 1 min and this caused the accumulation of proteins. (B) The analysis was done on 6% native gels stained with Coomassie R-250. The two ligand-receptor complexes, 1 and 2, are indicated. (C) The samples were electrophoresed under nonreducing conditions on 12% SDS minigels stained with Coomassie blue. (B, C) Lanes 1, Protein mixture loaded on the preparative gel. Lanes 2-30 (C, lanes 2-29), proteins eluted from the preparative gel. The fractions pooled are indicated. (C) Molecular mass markers (M) in kDa.

drical gels of 4 mm thickness and 5 cm height. The gel solution was overlayed with water-saturated isobutanol. After 2 h, the isobutanol was washed off and the 4% polyacrylamide stacking gel solution was poured into the cell to make 1-cm-high gel. The stacking gel was overlayed with isobutanol for 2 h, at which time the isobutanol was substituted with water and the gel was allowed to polymerize at room temperature overnight. The ligand and receptor solutions were separately concentrated by ultrafiltration to about 0.8 ml each. The mouse and human proteins were mixed at ligand-receptor ratios of 30/52 and 32/52 (weight/weight), respectively. Appropriate amounts were taken to prepare 10–20 mg of total protein mixture. The mixture was allowed to stay at 4°C for 30 min, and sample buffer (0.3 ml) was added and loaded on the gel. Electrophoresis was performed at 500 V under cooling with ice-cooled anode buffer (60 ml/min) for about 8 h. The proteins were eluted with cathode buffer at 60 ml/h and the elution

was followed by recording the absorbance at 280 nm. Three-milliliter fractions were collected and analyzed by SDS- and native polyacrylamide gels. The pooled fractions (60–100 ml) were concentrated by ultrafiltration (M_r , cutoff 10,000) to about 1 ml and the buffer was simultaneously exchanged to phosphate-buffered saline, pH 7.4.

RESULTS AND DISCUSSION

Our aim was to prepare IFN γ -IFN γ receptor complexes suitable for crystallization and biological mechanistic studies. At the same time, we investigated the possibilities of (i) removing most of the impurities from partially purified starting materials and (ii) removing the rest of the persisting impurities from highly purified starting materials.

IFN γ s were produced in *E. coli* in nonglycosylated form. The soluble IFN γ receptors were produced in baculovirus-infected insect cells and were heterogeneously

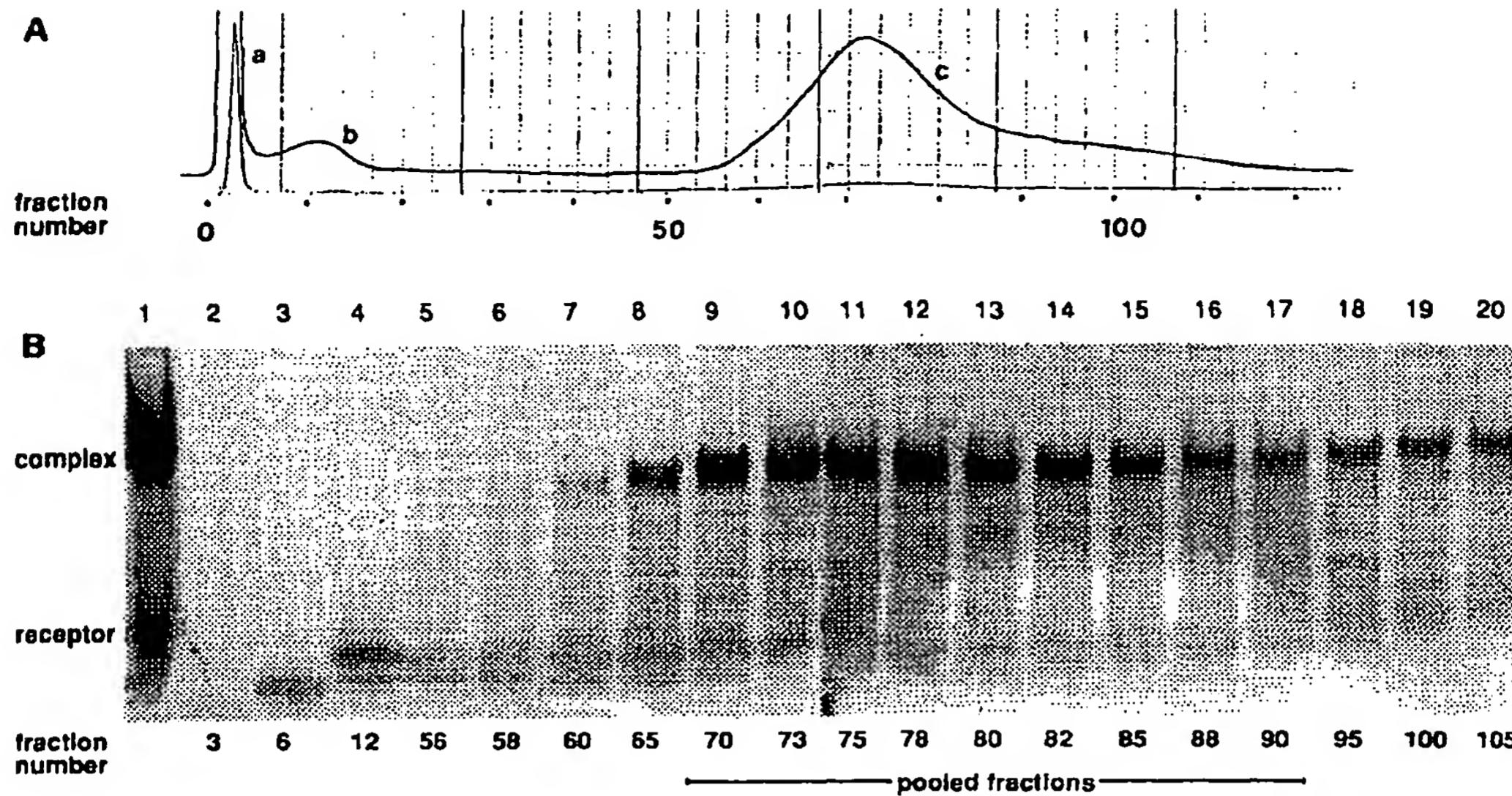


FIG. 4. Elution profile (A) and nondenaturing gel analysis (B) of the human IFN γ -IFN γ receptor complex eluted from the preparative native gel. (A) Absorbance recorded at 280 nm. (B) The fractions were electrophoresed on 6% analytical native gels stained with silver. Lane 1, Protein mixture loaded on the preparative gel. Lanes 2-20, Proteins eluted from the preparative gel. The fractions pooled are indicated.

glycosylated, the carbohydrate moieties contributing about 6 kDa to their apparent molecular masses. IFN γ s exist as dimers and IFN γ receptors as monomers in physiological buffer. One IFN γ dimer is bound by two receptor molecules, forming a complex of an apparent molecular mass of about 90 kDa (10).

In the first experiments, we used recombinant mouse IFN γ as ligand in our model. We used a side fraction of partially purified protein in which IFN γ constituted about 50% of total proteins (Fig. 1, lane 1). Because of very high proteolytic activity in the cell extract, IFN γ appeared as a partially digested doublet of 15 kDa (full-length) and 14 kDa (proteolytic product; not clearly seen in Fig. 1). The recombinant soluble mouse IFN γ receptor used here was also a side fraction, in which it constituted about 50% of total proteins (Fig. 1, lane 2). Because not all potential glycosylation sites were equally utilized, the receptor migrated as a complex of many bands at about 33 kDa (lane 2).

In the second set of experiments with highly purified proteins, human IFN γ and IFN γ receptor were used. IFN γ migrated as one band with an apparent molecular mass of 16 kDa (Fig. 1, lane 3) and the soluble human IFN γ receptor as three bands at about 32 kDa (lane 4). The 43-kDa band represents a persisting impurity whose percentage varied from batch to batch constituting 2-5% of the purified protein (lane 4). For a complete removal of this impurity, a large amount of the receptor

protein would have to be sacrificed, after the final sizing column, since the 43-kDa protein almost coeluted with the receptor. Even after mixing of the receptor with the ligand and chromatography of the mixture on a size exclusion column, complete separation of the 43-kDa contaminant could not be achieved in spite of the fact that the apparent molecular mass of the complex was about 90 kDa (Ref. 10; data not shown).

Preliminary studies with analytical, native slab gels had shown that IFN γ s and their receptors formed stable complexes. Heat-denatured components (95°C for 15 min) or reduced receptor did not form complexes (data not shown). In order to determine the proper concentration of the preparative polyacrylamide gels, three different concentrations of analytical, native gels were tried, representing 5, 6, and 7% acrylamide concentrations (Figs. 2A, 2B, and 2C, respectively). All three concentrations of acrylamide gave satisfactory separation of the complexes from the contaminating proteins, i.e., from the noncomplexed components and the accompanying impurities (Figs. 2A, 2B, and 2C, lanes 3 and 6). The mouse proteins formed two ligand-receptor complexes, a minor, faster migrating (designated 1) and a major, slower migrating (designated 2) complex (Figs. 2A, 2B, and 2C, lanes 3). Mouse IFN γ just entered the gel (Figs. 2A, 2B, and 2C, lanes 1). Human IFN γ did not give a clear band on the native gels (Figs. 2A, 2B, and 2C, lanes 4).

For the preparative gels, those acrylamide concentrations were chosen in which the ligand-receptor complexes migrated to the middle of the analytical gel, i.e., 6% for the mouse (Fig. 2B, lane 3) and 5% for the human (Fig. 2A, lane 6) proteins. In this way, prolonged electrophoresis and unnecessary dilution of the eluted proteins were avoided. For the preparation of the protein mixture, IFN γ s were considered as dimers of 30 kDa (mouse) or 32 kDa (human) and the IFN γ receptors as monomers of 26 kDa apparent molecular mass (glycosylation was not taken into account). Because one IFN γ dimer is bound by two receptor molecules (10), mouse and human proteins were mixed at a ligand:receptor ratio of 30:52 and 32:52 (on a protein weight basis), respectively. The mouse starting materials were considered 50% pure and the human starting components were considered over 98% pure. Corresponding amounts of ligand and receptor proteins were mixed to result in 10–20 mg of total protein loaded per gel.

Figure 3A shows the protein elution profile of the mouse proteins recovered from the preparative native gel. On account of the presence of many foreign proteins in the original protein mixture, no clear peak corresponding to the IFN γ -IFN γ receptor complex was resolved. The fractions were analyzed by native (Fig. 3B) and SDS-polyacrylamide gels (Fig. 3C). The first large peak, a (Fig. 3A), corresponded to the front and included the blue dye and some impurities (Figs. 3B and 3C, lanes 2). The following 50 fractions contained mainly impurities and noncomplexed ligand and receptor (Figs. 3B and 3C, lanes 3–10). The fractions of peak b contained both the faster 1 and the slower 2 migrating ligand-receptor complexes (Fig. 3B, lanes 11–20). The last fractions (Fig. 3A, peak c) contained almost only complex 2 (Fig. 3B, lanes 21–30). SDS-PAGE analysis showed that both complexes 1 and 2 contained ligand and receptor. The fractions which contained both complexes 1 and 2 included also some impurities (Figs. 3B and 3C, lanes 11–17), whereas only very small amounts of foreign proteins were present in the last fractions containing complex 2 (Fig. 3C, lanes 26–29). Thus, starting with partially purified proteins, the preparative electrophoresis provided us with a highly purified ligand-receptor complex. Moreover, it efficiently distinguished between the two kinds of ligand-receptor complexes. Chromatography on Sephadex G-100 did not discriminate between the two complexes. High-performance liquid chromatography showed the presence of two overlapping protein peaks, but the resolution was insufficient for separation (data not shown). This heterogeneity could be critical for the growth of diffractive crystals.

The ligand-receptor complex of the human proteins, eluting from the preparative gel, resolved to a distinct protein peak (Fig. 4A, peak c) because these proteins had already been purified before mixing and electropho-

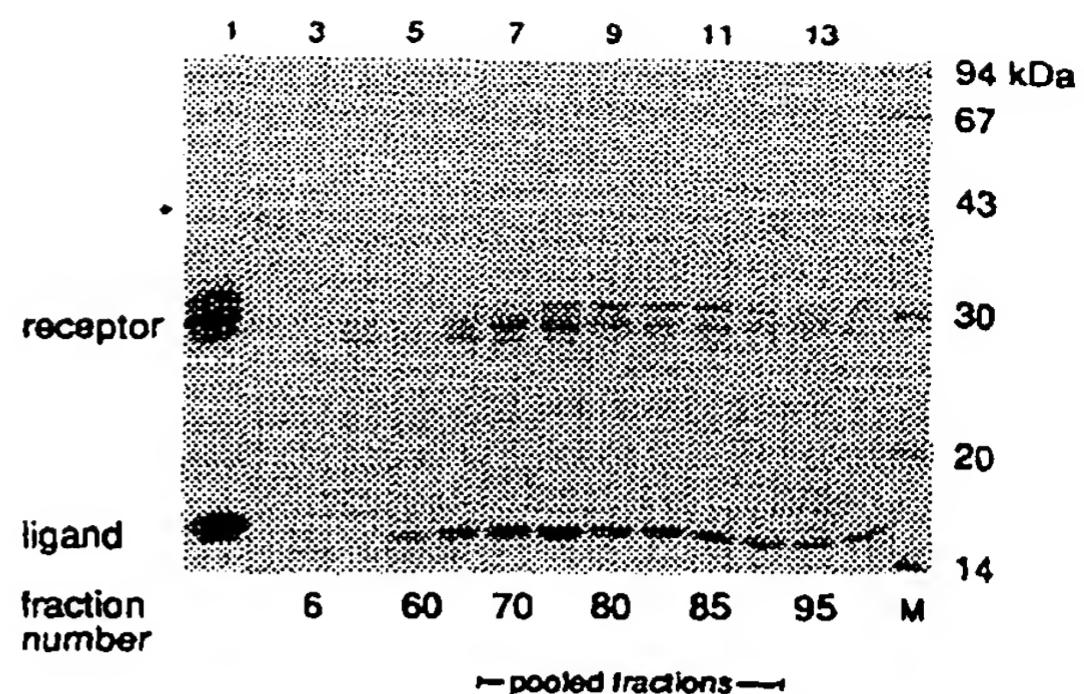


FIG. 5. SDS-PAGE analysis of the human IFN γ -IFN γ receptor complex eluted from the preparative native gel. The electrophoresis was performed on a 12% SDS-gel under nonreducing conditions. Lane 1, Protein mixture applied on the preparative gel. Lanes 2–14, Proteins eluted from the preparative gel. The bands of the receptor, the ligand, and the 43-kDa contaminant (*) are indicated. The receptor of lane 14 appears as a long, diffused band. This is due to the presence of β -mercaptoethanol in the neighboring slot with the molecular size markers (M) which causes this shift in mobility (Ref. 11). The fractions pooled are denoted.

resis. The first, large peak a (Fig. 4A), corresponded to the dye front. The second small peak, b, included the 43-kDa protein and noncomplexed soluble receptor (Fig. 4B, lanes 3 and 4; Fig. 5, lanes 2–4). Analysis of the fractions from peak c (Fig. 4A), by native gels, showed that a small amount of the receptor was separated from the complex in all fractions (Fig. 4B, lanes 7–20). This is probably due to the interchange of the ligand and receptor molecules during electrophoresis.

SDS-polyacrylamide gel analysis of fractions in peak c showed the presence of both ligand and receptor (Fig. 5, lanes 5–14). The heterogeneous glycosylation of the receptor probably influenced the charge of the molecules and subsequently their migration on the native preparative gel. The fractions near the beginning (Fig. 5, lanes 5 and 6) and near the end (lanes 13 and 14) of the peak, which contained receptor with glycosylation patterns substantially different from those in the middle fractions (lanes 7–12), were not pooled, so that a more homogeneous ligand-receptor complex was recovered. Unlike electrophoresis, size exclusion chromatography did not efficiently discriminate on the basis of the oligosaccharides present (not shown).

After analysis by native and SDS-polyacrylamide gels, the fractions containing the ligand-receptor complex were pooled and concentrated by ultrafiltration to the desired final volume. Concentration was necessary because during electrophoresis a significant dilution of the protein solution took place (loaded protein volume on the gel about 2 ml; volume of the pooled fractions

TABLE I
Amino Acid Composition Analysis of Mouse and Human IFN γ s and Their Receptors^a

Amino acids	Residues in protein							
	IFN γ				IFN γ Receptor			
	Mouse		Human		Mouse		Human	
	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed
Lys	10	8.2	20	19.5	15	14.7	14	13.0
His	3	2.6	2	2.0	6	5.7	5	4.6
Arg	6	5.1	5	4.7	8	7.8	6	6.3
Trp	2	N.D.	1	N.D.	4	N.D.	4	N.D.
Asx	17	14.8	20	20.2	23	23.1	26	25.3
Thr	3	2.6	5	3.9	16	13.7	11	9.2
Ser	15	10.7	10	8.6	23	18.9	20	15.7
Glx	17	15.4	17	18.0	27	28.1	29	29.1
Pro	2	2.1	2	0.9	13	14.9	12	12.3
Gly	3	3.8	4	4.5	11	11	12	11.8
Ala	5	5	7	7	5	5	8	8
1/2Cys	1	N.D.	0	—	10	N.D.	8	N.D.
Val	8	6.6	8	7.6	24	21.7	25	22.9
Met	3	2.6	3	3.0	4	4.0	5	3.9
Ile	12	9.4	7	6.5	11	10.6	20	18.0
Leu	13	11.2	9	8.8	12	11.5	7	7.0
Tyr	2	1.8	4	3.9	10	9.7	12	10.9
Phe	9	6.7	9	9.3	7	6.6	5	5.2

Note. The calculations were done according to the least-square method of Antoni and Presentini (4). The observed amino acid ratios of the components and their corresponding complexes were introduced in the computer program. The deviations of the found ligand-receptor molar ratios from integral values are within the experimental error limits. The molar amounts and ratios found are: Mouse proteins: (i) complex 2 in solution: IFN γ dimer, 0.054 nmol; IFN γ receptor, 0.095 nmol. This gives a concentration ratio of IFN γ dimer-IFN γ receptor 1.00:1.78, or, one IFN γ dimer associated with two IFN γ receptors. (ii) complex 1 (band cut from the PVDF membrane): IFN γ dimer, 2.10 pmol; IFN γ receptor, 2.62 pmol. IFN γ dimer-IFN γ receptor ratio 1.00:1.25, or one IFN γ dimer associated with one receptor. (iii) complex 2 (band cut from the PVDF membrane): IFN γ dimer, 0.68 pmol; IFN γ receptor, 1.49 pmol. Molar ratio 1.00:2.19, or one IFN γ dimer bound by two IFN γ receptors. Human proteins: IFN γ dimer 0.51 nmol; IFN γ receptor 0.96 nmol. IFN γ dimer-IFN γ receptor 1.00:1.88, or one IFN γ dimer associated with two IFN γ receptors.

^a The stoichiometry of ligand-receptor interaction was determined by amino acid composition analysis (3). The observed values are those of the components before mixing and are compared with the predicted ratios. The values found for the complexes are not shown. No corrections of losses on account of hydrolysis were made. The values were normalized to the Ala residues. N.D., Not determined.

60–100 ml). The recovery yield of pure ligand-receptor complexes was 30–40%. For the human proteins, this relatively low yield was partially due to the selection of only those fractions that contained receptor with similar glycosylation patterns. In the case of the mouse proteins, two kinds of ligand-receptor complexes were formed but only the slower migrating complex 2 was taken into account. We did not examine the presence of acrylamide in the eluted protein solution, but the existence of trace amounts would not influence the use of the complexes in the studies mentioned.

We further tested whether the ligand and receptor in the complexes recovered from the preparative native gels retained the proper stoichiometry of interaction of one IFN γ dimer bound by two receptor molecules (10). The stoichiometry of binding was determined by amino acid composition analysis (3,4). The pooled fractions of the mouse proteins (Fig. 3B, lanes 21–27; complex 2) contained IFN γ dimer and receptor at a molar ratio of

1:1.78 suggesting that mainly one IFN γ dimer is bound by two receptors. Because the mouse proteins formed two kinds of complexes (1 and 2, Fig. 3B), samples of selected fractions were electrotransferred to a PVDF membrane and the bands corresponding to the two complexes were cut out and subjected to amino acid analysis. IFN γ dimer and receptor of complex 1 had a molar ratio of 1:1.25, suggesting a one IFN γ dimer-one receptor binding mode. The IFN γ dimer-receptor ratio of complex 2 was 1:2.19, indicating that one IFN γ dimer is bound by two receptor molecules (Table 1). For the human proteins, a ligand dimer-receptor molar ratio of 1:1.88 was found which also corresponds to two receptors per ligand dimer (Table 1).

The results reported here, concerning the stoichiometry of binding (ligand dimer-receptor modes of 1:1 and 1:2 for the mouse, and 1:2 for the human proteins), agree with those previously reported (7,10). In previous studies, cross-linking experiments with the mouse proteins

had shown the presence of a 60-kDa complex, compatible with one IFN γ dimer bound by one receptor molecule (7,10). Ultracentrifugation analysis had shown a middle value, suggesting the existence of the two binding modes (10). For the human proteins, all approaches applied had shown mainly one binding mode of 1:2 (10).

Purification of protein-protein complexes by electrophoresis on preparative native gels has several advantages: (i) it is relatively simple and eliminates the necessity of performing other methods, such as the use of sizing columns to prepare the complexes and, most importantly, the development of purification steps in order to obtain pure components before mixing; (ii) it is superior to size exclusion chromatography by providing higher homogeneity; (iii) the isolated complexes can be directly used in determination of the stoichiometry of interaction between ligand and receptor; (iv) it facilitates the long process of structure-based, rational drug design by substantially shortening the protein purification time. In the cases presented here, purification of mouse IFN γ and removal of the 43-kDa impurity of the human receptor had been laborious and costly. The presence of foreign proteins, on the other hand, might be inhibitory for the crystallization of the high molecular mass complexes; (v) it appears to be a general approach for preparing protein-protein complexes, starting from semipurified materials, as stable complexes are formed not only between ligands and receptors, but also between receptors and antibodies against them or F_{ab} fragments (tested for the human system; data not shown). In the latter case, the design of antagonists against surrogate ligands could be facilitated.

In conclusion, we prepared pure mouse and human IFN γ -IFN γ receptor complexes in one step using pre-

parative electrophoresis on nondenaturing gels. We achieved a significant purification factor by removing the remaining or persisting impurities from partially or highly purified starting protein mixtures, respectively. The method may be of general interest for purification of protein-protein complexes.

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